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Boulder, Colorado 80309

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Telomeres are the nucleoprotein structures that cap the ends of eukaryotic chromosomes. Telomere length is controlled by the enzyme telomerase and a suite of telomere binding proteins. Anomalous telomeric replication and regulation are implicated in most forms of cancer, while telomeric shortening contributes to cellular aging. Cdc13p is an essential protein from *S. cerevisiae* that binds to the single-stranded ends of telomeres with high specificity and affinity. Genetically, Cdc13p has been shown to protect the end of the chromosome from degradation and to load telomerase in concert with the protein Est1p. Biochemically, Cdc13p binds yeast single-stranded telomeric DNA (sstelo DNA) *in vitro* with high affinity (K_d=0.3 nM). The DNA-binding domain of the protein has been mapped previously.

We are investigating the structural and biochemical basis for high affinity binding and sequence specificity of this domain. A high resolution solution structure of the protein/DNA complex is in progress. Here we use NMR experiments to determine the single-stranded DNA conformation in the complex and directly observe protein/DNA NOE contacts. In a complementary approach, we have performed *in vitro* protein/DNA photocrosslinking experiments using the chromophore 5-iodouracil. Proteolytic digestion and peptide micro-sequencing have allowed identification of sites in the protein involved in binding ssDNA.

14. SUBJECT TERMS
breast cancer, telomeres, telomerase, single-stranded DNA binding protein, Cdc13, nuclear magnetic resonance (NMR), structural biology, biophysical chemistry, structure-function relationships, *Saccharomyces cerevisiae*

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Annual Report for: Understanding Single-Stranded Telomere End Binding by an Essential Protein

Emily M. Anderson
Department of Chemistry and Biochemistry
University of Colorado at Boulder

TABLE OF CONTENTS

INTRODUCTION

Telomeres are the nucleoprotein complexes that protect the ends of linear eukaryotic chromosomes. Telomere replication and length regulation are controlled by the enzyme telomerase and a suite of telomere binding proteins. Anomalous telomeric replication is implicated in most forms of human cancer. Telomere metabolism is thus an active field in basic research for the eventual goal of developing inhibitors or modulators of telomere replication for cancer therapy. Cdc13p is an essential protein from the budding yeast *Saccharomyces cerevisiae* whose role is to protect the end of the chromosome from degradation and to load telomerase in concert with the protein Est1p. Biochemically, Cdc13p binds to single-stranded yeast telomeric DNA with high affinity and specificity. We are investigating the structural basis for high affinity binding and sequence specificity of the DNA binding domain. One aspect of this research involves solving the high resolution solution structure of the domain complexed to DNA using heteronuclear, multidimensional NMR. Biochemical techniques are also being employed, including mapping regions of the domain in proximity to the DNA by photocrosslinking and investigating sequence specificity using libraries of DNA with varying sequences. The advantage of studying this protein using yeast as a model organism is the power of combining structure, biochemistry, and genetics all in one system.

BODY

Significant progress toward accomplishment of the technical goals has been made in the last year Technical objective 1, outlined below, was completed in full as of the report submitted one year ago.

Technical Objective 1:

Express and purify DNA binding constructs	2 Months
Conduct binding assays with site-randomized DNA	4 Months
Conduct CD experiments of protein folding and DNA binding	1 Month

An optimized DNA-binding domain construct has been delineated using proteolysis and MALDI mass spectrometry. This construct has been subcloned, expressed and purified in high yield, suitable for high resolution structural characterization. The construct binds DNA with affinity comparable to that reported for the full-length protein as measured by both gel-shift binding assays and nitrocellulose filter-binding assays. Binding assays were conducted with site-randomized single-stranded DNA oligomers to determine bases in the DNA critical for binding affinity and specificity. These experiments are to be followed up by experiments involving chemical modification of the DNA with dimethylsulfate. Circular Dichroism experiments were performed to assess the secondary structural content of the domain, whether there are gross structural changes upon DNA binding, and to assess the thermostability of the domain in isolation. It was found that the domain in isolation forms a compact, stable, globular structure with both α helical and β sheet structure content. No major secondary structural changes occurred upon DNA binding

Technical objective 2 is also essentially complete, as outlined below.

Technical objective 2:

Conduct photocrosslinking/identify contacts	3 Months
Design mutants/test *in vitro* and *in vivo*	6 Months

Photocrosslinking experiments with the chromophore iodouracil substituted for thymine have been performed. The DNA substrates used are outlined in Figure 1.

Name	Sequence
DNA1	dG^{I}UGTGGGTGTG
DNA2	dGTG^{I}UGGGTGTG
DNA3	dGTGTGGG^{I}UGTG
DNA4	dGTGTGGGTG^{I}UG

Figure 1. Sequences of the four modified single-stranded DNA oligomers used in the photocrosslinking study. ^{I}U represents the chromophore iodouracil.

The peptide in the domain which crosslinks to DNAs 2,3, and 4.has been identified using trypsin digestion and micro-Edman sequencing. The sequence of the entire domain is given in Figure 2, along with the crosslinked peptide in bold. Trypsin skips one possible cut site between lysine26 and tyrosine27 when the domain is covalently bound to DNA. Edman sequencing did not identify the precise amino acid in the peptide bound to DNA, as N-terminal sequencing is only possible for the first 20-25 amino acids of this 46 amino acid peptide. In two cases, threonine 25 and threonine 29 were recorded as "blanks" in the sequence implicating them as sites of crosslinking. However, due to the size of the peptide, complete sequences could not assign them as the crosslinking sites wth complete confidence. Mass spectrometry was also not successful in identifying the amino acid involved in the crosslink. However, this peptide is completely in agreement with the region of the protein involved in DNA binding as revealed by structural studies (see technical objective 3). As specific amino acids were not identified using this technique, mutagenesis to analyze the DNA-binding interface is being designed in conjunction with the data collected in technical objective 3.

MRMSKMAR**KD PTIEFCQLGL DTFETKYITM FGMLVSCSFD KPAFISFVFS DFTK**NDIVQN
YLYDRYLIDY ENKLELNEGF KAIMYKNQFE TFDSKLRKIF NNGLRDLQNG RDENLSQYGI
VCKMNIKVKM YNGKLNAIVR ECEPVPHSQI SSIASPSQCE HLRLFYQRAF KRIGESAISR
YFEEYRRFFP IHRNGSHLA

Figure 2. Primary sequence of the Cdc13p ssDNA binding domain with the crosslinked tryptic peptide in bold.

Technical objective 3 involves determining the high resolution NMR solution structure of the domain. This objective is also well on its way to completion. As stated in last year's annual progress report, the focus of the research until now has been on the protein/DNA complex with the collaboration of another student in the laboratory, Rachel Mitton-Fry. The original Technical Objective 3 is listed as follows:

Technical Objective 3:

Optimize solution conditions of sample for NMR spectroscopy	1 Month
Protein alone –	
Collect heteronuclear NMR data for resonance assignment	6 Months
Assign resonances in the protein domain	6 Months
Collect heteronuclear NMR data for distance restraints	1 Month
Determine family of structures that satisfy restraints	6-12 Months
Protein/DNA complex –	
Titrate DNA into protein and conduct NMR experiments	6-18 Months

Solution conditions were optimized and many of the heteronuclear NMR experiments were conducted on the complex before submission of last year's annual report. Resonance assignments were also well on their way to completion. A ^{15}N-labeled sample of protein was prepared and used to titrate with DNA; the complex is in slow-exchange on the NMR time scale. This data is presented in Figure 3.

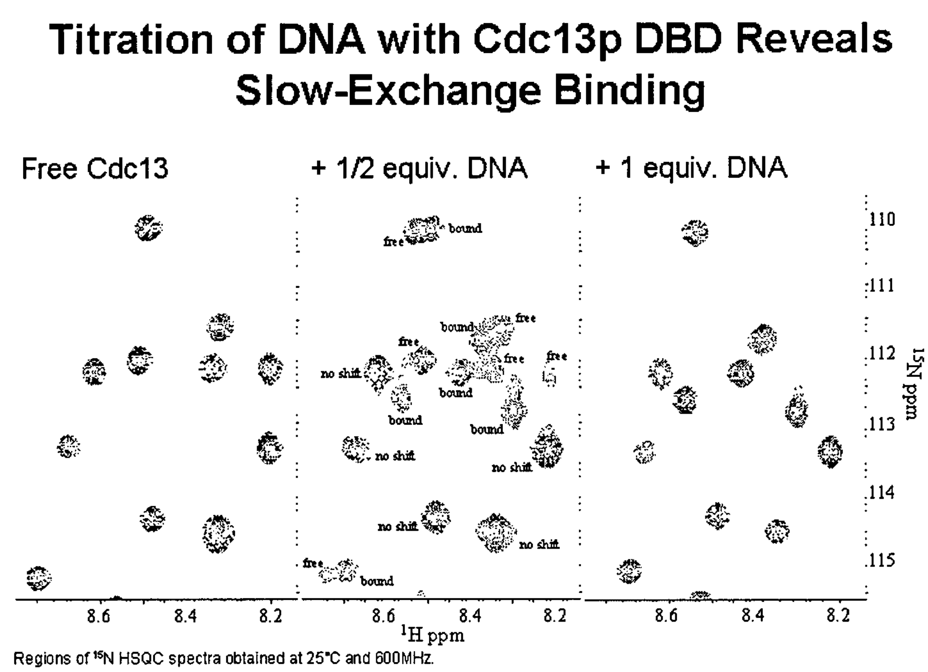

Figure 3. A selected region of ^{15}N HSQC spectra of Cdc13p DNA binding domain with increasing amounts of dGTGTGGGTGTG added.

This year, protein resonance assignments of the protein/DNA complex have been essentially completed by Rachel Mitton-Fry. Preliminary structures of complex have been generated and are being refined with the addition of remaining NOE restraints and dihedral angle restraints from 3-bond scalar coupling measurements. The domain has a mixed α/β topology and contains a β-barrel type fold.

In our preparations of the complex the DNA is unlabeled and is not observed in the isotope-selected experiments conducted so far. This year I have performed isotope-filtered NMR experiments to examine the unlabeled single-stranded DNA in the complex and it appears to be in an extended conformation. The DNA exists in a unique conformation with 11 identifiable spin systems. To aid in assignment of the spin systems I will conduct experiments with various thymine bases substituted with uracil. Site-specific ^{13}C-labeled samples may also be prepared. Also I have conducted isotope select-filter experiments to measure NOE contacts between the protein and DNA. From this data we have mapped a DNA-binding interface or cleft on the preliminary protein structure which is consistent with other measurements on the complex such as: chemical shift changes that occur upon binding, protection from hydrogen exchange, and mapping of a net positively-charged groove on the surface of the protein. Currently mutations are being chosen to test the thermodynamic contributions of these interface residues to binding

We have discovered that a construct of the domain with a C-terminal 6-Histidine tag is significantly more soluble in the absence of DNA than the construct lacking the His-tag (whose solution lifetime was about 12 hours under the best circumstances). This finding will allow the structure of the free protein to be determined which would not have been possible before. A ^{15}N-labeled NMR sample of this His-tagged construct was prepared without DNA and lasted several weeks in solution, which is long enough to make preparation of ^{15}N,^{13}C-labeled samples feasible and conduct the triple-resonance experiments required for structural study.

. It should be noted that some of the subtasks in technical objective 3 are being completed in parallel by myself, while some are being completed by Rachel Mitton-Fry. In this respect completion of the entire project, which has a total time frame of 5 years, should be completed well within the scope of the granting period, which lasts another year for a total of 3 years of funding.

KEY RESEARCH ACCOMPLISHMENTS (THIS YEAR)

- A specific peptide in the N-terminal region of the domain was identified as photocrosslinking to several iodouracil-modified DNAs. This peptide localizes to the protein/DNA interface as calculated by structural methods.
- Titration of the protein with DNA has revealed that the complex is in slow exchange on the NMR timescale.
- Assignment of protein NOE crosspeaks in the complex (distance retraints) is nearly complete.
- Preliminary structures of the protein in the complex have been calculated.
- Double-filtered isotope experiments have revealed that the bound DNA is in an extended conformation.
- Select-filter isotope NOE experiments have delineated a set of residues that contact DNA which form a DNA-binding interface on the surface of the protein.
- Mutations have been designed to measure the thermodynamic effect of residues at the DNA-binding interface.
- A 6-His tagged version of the protein domain was found to be significantly more soluble than without the tag, allowing structural studies of the domain in the absence of DNA.

REPORTABLE OUTCOMES

Abstracts: The work in progress has been presented as a poster at two meetings: Telomeres and Telomerase (Cold Spring Harbor, NY), and the 15th Symposium of the Protein Society (Student Poster Award – Philadelphia, PA).

Presentations: This work has been presented as a talk at the University of Colorado Biophysical Club in May, 2001.

Abstracts of papers presented
at the 2001 meeting on

TELOMERES & TELOMERASE

March 28–April 1, 2001

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

STRUCTURAL AND BIOCHEMICAL INVESTIGATION OF SINGLE-STRANDED TELOMERIC DNA BINDING BY CDC13P

E.M. Anderson[1], R.M. Mitton-Fry[1], T.R. Hughes[2], V. Lundblad[2], D.S. Wuttke[1]
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Cdc13p is an essential protein from *S. cerevisiae* that binds to the single-stranded ends of telomeres with high specificity and affinity. Genetically, Cdc13p has been shown to protect the end of the chromosome from degradation and to load telomerase in concert with the protein Est1p.[1,2] Biochemically, Cdc13p binds yeast single-stranded telomeric DNA (sstelo DNA) *in vitro* with high affinity (K_d=0.3 nM). The DNA-binding domain of the protein has been mapped by deletion analysis and proteolysis.[3]

We are investigating the structural and biochemical basis for high affinity binding and sequence specificity of the single-stranded DNA binding domain. A high resolution solution structure of the complex is in progress in our laboratory. Heteronuclear, multidimensional NMR and ^{15}N and ^{13}C isotopic labeling of the protein domain have allowed us to make complete resonance assignments of the protein (see poster presented by RMF). Here we report the use of filtered and selected/filtered NOE experiments to determine the single-stranded DNA conformation in the complex and directly observe protein/DNA NOE contacts. We have also performed *in vitro* protein/DNA photocrosslinking experiments using the chromophore 5-iodouracil substituted for thymine in the DNA. Proteolytic digestion of the crosslinked products along with peptide micro-sequencing and ESI mass spectrometry allowed identification of sites in the protein involved in sstelo DNA binding.

1. Nugent, C.I., Hughes, T. R., Lue, N. F., Lundblad, V., (1996) *Science*, 274, 249-252.
2. Evans, S.K., Lundblad, V., (1999) *Science*, 286, 117-120.
3. Hughes, T.R., Weilbaecher, R. G., Walterscheid, M., Lundblad, V., (2000) *Proc. Natl. Acad. Sci.*, 97(12), 6457-6462.

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the hydroxyl grou[illegible]) that the rate of the
overall reaction [illegible] the conversion of
aminoacrylate to [illegible]ompare our results
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346

Self-associatio[illegible] half of human RAD52 and i[illegible]uman replication protein A

Doba D. Jacks[illegible]a Ranatunga[1], and
Kendall L Knig[illegible]

[1]University of [illegible]t st., Toledo, OH
43606, [2]Univer[illegible]

The human R[illegible] play an important
role in the ear[illegible]omal double-strand
break repair v[illegible]mbination pathway.
Individual sub[illegible] self-associate into
rings that can [illegible]complexes. Human
RAD52 (hRA[illegible] bind human RPA
(hRPA) and [illegible]nown to stimulate
homologous [illegible]oting single-strand
annealing an[illegible]ys. Earlier studies
defined the s[illegible] hRAD52 to the N-
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the hRAD5[illegible]nserved domains on
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with hRAD[illegible]d in promoting single-
strand anne[illegible] pathways in the cell.

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347

Structural and Biochemical Studies of Single-Stranded Telomeric DNA Binding by an Essential Protein

Emily M Anderson[1], Rachel M Mitton-Fry[2], Timothy R Hughes[3], Victoria Lundblad[3], and Deborah S Wuttke[1]

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Telomeres are the nucleoprotein structures that cap the ends of eukaryotic chromosomes. Telomere length is controlled by the enzyme telomerase and a suite of telomere binding proteins. Anomalous telomeric replication and regulation are implicated in most forms of cancer, while telomeric shortening contributes to cellular aging. Cdc13p is an essential protein from *S. cerevisiae* that binds to the single-stranded ends of telomeres with high specificity and affinity. Genetically, Cdc13p has been shown to protect the end of the chromosome from degradation and to load telomerase in concert with the protein Est1p. Biochemically, Cdc13p binds yeast single-stranded telomeric DNA (sstelo DNA) *in vitro* with high affinity (K_d=0.3 nM). The DNA-binding domain of the protein has been mapped by deletion analysis and proteolysis.

We are investigating the structural and biochemical basis for high affinity binding and sequence specificity of the single-stranded DNA binding domain. A high resolution solution structure of the protein/DNA complex is in progress in our laboratory. Here we report the use of NMR experiments designed to determine the single-stranded DNA conformation in the complex and directly observe protein/DNA NOE contacts. In a complementary approach, we have performed *in vitro* protein/DNA photocrosslinking experiments using the chromophore 5-iodouracil. Proteolytic digestion along with peptide micro-sequencing and ESI mass spectrometry allowed identification of sites in the protein involved in sstelo DNA binding.

We thank the NIH, the American Cancer Society, a CU Junior Faculty Development Award, a HHMI Predoctoral Fellowship (RMF), and the US Army Breast Cancer Research Program (EMA) for funding.

348

Kinetics of yTBP binding with Biotinylated TATA DNA by Biacore Analysis

Lumelle A Schneeweis[1], Michael R Brigham-Burke[1], and B Franklin Pugh[2]

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The TATA-binding protein (TBP) recognizes the TATA box sequence of the promoter region of eukaryotic genes. This interaction is critical for the recruitment and assembly of the eukaryotic transcription initiation complex. The recruitment of TBP at a promoter is thought to be rate-limiting in transcription of RNA polymerase II-transcriped genes, and thus an important regulatory point. The mechanism of TBP binding to the TATA-box recognition site on DNA is important for understanding eukaryotic transcription regulation. This interaction has been studied by Biacore surface plasmon resonance analysis through the capture of biotinylated TATA-containing DNA on the sensor surface and solution binding of yTBP.

DEPARTMENT OF THE ARMY
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